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Original Research

DADOS Spectroscopy of Organotypic Brain Slices to Determine Hydrogen Peroxide-Induced Oxidative Stress

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Abstract

Background: Organotypic brain slices are state-of-the-art *ex vivo* preparations that allow the study of all types of brain cells for several weeks to months. The brain slices are cultured on semi-permeable membranes, and as the slices flatten and become transparent, remain viable for several weeks. However, without cellular staining, assessing slice health remains challenging. Thus, this study aimed to determine whether assessing spectroscopy of the living brain slices can detect oxidative stress. **Methods:** Brain slices (300 µm) were prepared from postnatal day 8–10 mouse brains and cultured as quarter-brain slices at the hippocampal level. Spectrography was performed using an adapted DADOS spectrograph. Fresh, living slices were tested with exposure to three laser wavelengths: red-green-blue, ultraviolet (UV), and infrared (IR) light, and a neon and mercury lamp. To induce oxidative stress, some slices were treated with 9.7 mM hydrogen peroxide for 2 days, and others were then stained with the red fluorescent nuclear cell death dye propidium iodide. **Results:** Our data showed that the DADOS spectrograph can detect spectra in living brain slices using all seven light sources. PI treatment alone decreased the detected wavelength signal when exposed to mercury, but increased it when exposed to neon. Hydrogen peroxide reduced the wavelength after exposure to IR and the red laser, while the area (intensity) was enhanced after exposure to UV, mercury, neon, and IR. **Conclusion:** Our data show that oxidative stress-mediated processes can be measured with a DADOS spectrograph in living organotypic brain slices. The slices showed a differential spectral pattern when stimulated with seven different light sources. This study pioneers a novel, low-cost, easy-to-use DADOS spectroscopy tool for living brain slices.

Keywords: organotypic brain slice; DADOS spectroscopy; hydrogen peroxide; oxidative stress

1. Introduction

Organotypic brain slices are 3-dimensional preparations that preserve all major brain cell types within intact complex networks and can be cultured for several weeks. The culturing of organotypic brain slices has been pioneered in our lab for nearly 25 years, for the basic and experimental research on neurons, along with astrocytes, microglia, and vessels [1,2,3]. The methodological expertise is reflected in the high-quality preservation of brain slices under defined control conditions, which can be cultured for several weeks. Two crucial factors in the culturing and study of organotypic brain slices are, first, a slice thickness of 150 µm, which proved to be optimal for survival, and for later microscopic visualization. Second, the age of the donor animal is a critical factor influencing the quality of organotypic brain slices. In our lab, we use postnatal day 8–10 mice, as slices obtained at this developmental stage exhibit superior morphology and viability compared to slices from older donors. The quality of the stored slices can be validated by visual appearance, such as viable slices fully attaching to the membrane and appearing thinner and more transparent over time. This can also be verified at the end of culturing by the blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining [4], which mainly shows a clear homogeneous cell layer all over the brain slice. In addition to this staining, cell death assays are also performed, such as propidium iodide (PI) on living slices, which should not result in many red fluorescent nuclei in the healthy brain tissue. PI selectively stains dead cells, as it cannot penetrate an intact plasma membrane [5], and many PI+ nuclei are found during cell death. Finally, the ultimate test for the viability of neurons, astrocytes, or microglia is determined by cell-specific markers using immunohistochemical staining. The slice model also allows for the co-staining of different structures with several fluorescent dyes (blue-green-red fluorescent) to provide insights into the three-dimensional tissue organization.

Spectroscopy is the analysis of light after separation with a diffractive element, giving a light spectrum [6]. Normal light can be seen with our eyes between a wavelength of 400 nm (violet) and red (750 nm), while ultraviolet light (UV) and infrared light (IR) show wavelengths in the blue (100–400 nm) and red (>780 nm) wavelength range, respectively. The different light wavelengths can be visualized with a spectrograph, resulting in a spectrogram with many different wavelength peaks and intensities when separated by a diffractive element. Spectroscopy has a wide range of uses in chemistry to identify substrates, in medicine for

tissue and organ visualization, in the environment for testing toxic substances, or in material science. The principle is that light hits a substrate, is absorbed and reflected, and the emitted light is altered by the structure. Many different forms of spectroscopy have been used and established in basic science, most prominently Raman spectroscopy [7,8] and fluorescence spectroscopy [9,10], and electrical impedance spectroscopy [11], but also electron paramagnetic resonance spectroscopy, near-infrared spectroscopy, magnetic resonance spectroscopy or polarized light spectroscopy. These systems are complex and expensive. The focus of tissue examination is mainly on neoplastic tissue and cancer [10] or ocular retina or cornea [9,11] or skin epidermal tissue [7,8] but also on bone and osteogenesis [12] and some studies on brains. One study tested oxidative stress in organotypic brain slices using electron spin resonance spectroscopy, aiming to develop new drugs [13]. Other studies used an Alzheimer-like model to test okadaic acid-induced hyperphosphorylation with impedance spectroscopy [14,15]. Another study tested inflammation in a murine model of organotypic hippocampal slices with electron paramagnetic resonance spectroscopy [16]. In a human organotypic postmortem model of the corpus callosum, Raman spectroscopy was used to test altered myelin spectral profiles [17]. A very early study used hippocampal organotypic brain slices to study the metabolism of calcium with electron spectroscopic imaging [18]. And brain mitochondrial redox state was measured with Raman spectroscopy in organotypic cortical slices in a diabetic model after high glucose treatment [19]. Definitely, spectroscopy has great power to determine metabolic processes in living brain slices.

The DADOS spectrograph has been developed for astronomy [20], has 3 slits and a resolution up to 1200 lines/mm. Thus far, no DADOS spectroscopic analysis has been performed for organotypic brain slices as a whole, in living tissues. Thus, the rationale of the present study is to test if DADOS spectroscopy could be an alternative to other spectroscopic methods, as it is a cheap and easy system (costing approximately 5000 euros) and can be easily connected to the brain slice technology. We hypothesize that the spectrum of the brain slice is altered by oxidative stress in living organotypic brain slices. In order to induce oxidative stress in brain slices, we used the very easy and well-established hydrogen peroxide (H_2O_2) model [21,22].

The present study aimed to set up and optimize, for the first time, the spectrographic analysis of living organotypic brain slices using a DADOS spectrograph. We demonstrated the optimization of the spectrographic analysis in seven different lights: the lasers red-green-blue, UV, and IR light, as well as neon (Ne) and mercury (Hg) light. Using hydrogen peroxide, we will show that distinct bands and intensities are altered, pointing to oxidative stress-related processes.

2. Materials and Methods

2.1 DADOS Spectrograph

To perform spectrography of brain slices a DADOS spectrograph (Nr. 2458550, Baader, Mammendorf, Germany) with a reflection grating of 200 lines/mm and 3 slits (25–35–50 µm) was used and connected to an ASI322 MM mono camera (Nr. 75717, ZWO, Baader, Mammendorf, Germany) via an ASI air computer (Nr. 77072, ZWO, Baader) connected to an iPAD (Fig. 1). A modified adapter with a height of 2 cm and with a 1 mm hole (Miller Optics, Innsbruck, Austria) was placed into the "light opening" of the spectrograph.

2.2 Light Source for Stimulation

Three different lasers were used (all 5 mW from Starlight, laser Class 3R), a UV light (270 nm, 00 LM, 4400 mAh, stier.de, Germany), an infrared light (YZY=E, X001T11UX7, Powerstyle, China), as well as a neon lamp (Nr. 2458590; Baader, Germany) and a mercury lamp (Zeiss UHW50f-2b, 50–60 Hz, Miller Optics Innsbruck). The spectrograph shows a typical lane in the normal LED light (Fig. 2A), a single blue lane at 405 nm (Fig. 2B), a single green lane at 532 nm (Fig. 2C), and a single red lane at 605 nm (Fig. 2D). The neon lamp gives several lines (Fig. 2E), while four major distinct lanes are seen in the mercury lamp (Fig. 2F).

2.3 Organotypic Brain Slices

Organotypic brain slices were prepared as per the standardized protocols established in our lab [1,2,3]. Shortly after postnatal day 8–10 C57BL/6 wild-type mice (male and female) were decapitated, with no exclusion criteria (sample size n = 8–12), and the brains were taken. Coronal 300 µm thick brain slices were sectioned with a Vibratome (Leica Microsystems, Wetzlar, Germany) at the hippocampal level, and a quarter slice with the hippocampus (Fig. 3B) was placed on a 1 × 1 cm Isopore™ 0.4 µm pore PC membrane (HTTP02500, Merck Millipore, Burlington, MA, USA). These membranes with the slice were transferred onto semipermeable 0.4 µm pore cell culture inserts (PICM03050, Merck Millipore), which were placed into 6-well-plates (Greiner Bio-One, Kremsmünster, Austria). Each well contained 1.1 mL of sterile-filtered culture medium (50% MEM/HEPES (Gibco, Carlsbad, CA, USA), 25% Hanks' solution (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech, Austria), 6.5 mg/mL glucose (Merck, Germany), 2 mM $NaHCO_3$ (Merck, Austria), and 2 mM glutamine (Merck, Germany), at pH 7.2). The brain slices were cultured at 37 °C with 5% CO_2 for 2 weeks, and the culture medium was changed once a week. After culturing for 2 weeks, the living slices were put into the adapter (Fig. 3A), the hippocampus was positioned over the hole, and then exposed (Fig. 3C).

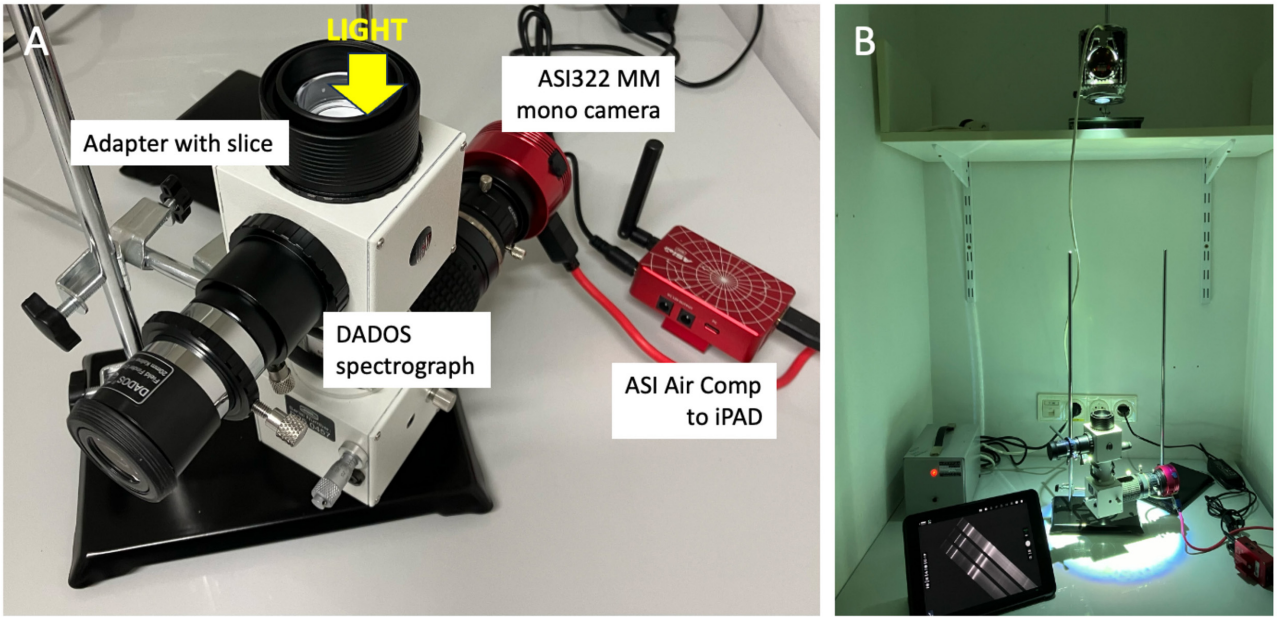

Fig. 1. Set-up of a DADOS spectrograph. The DADOS spectrograph is connected to a camera connected to an iPad via Bluetooth and an ASI air computer (A), and the light is beamed to the lens from above (yellow arrow) (A) and goes through an adapter to the DADOS (B). The complete setup is seen in Fig. 1B, where a mercury lamp is positioned 60 cm above.

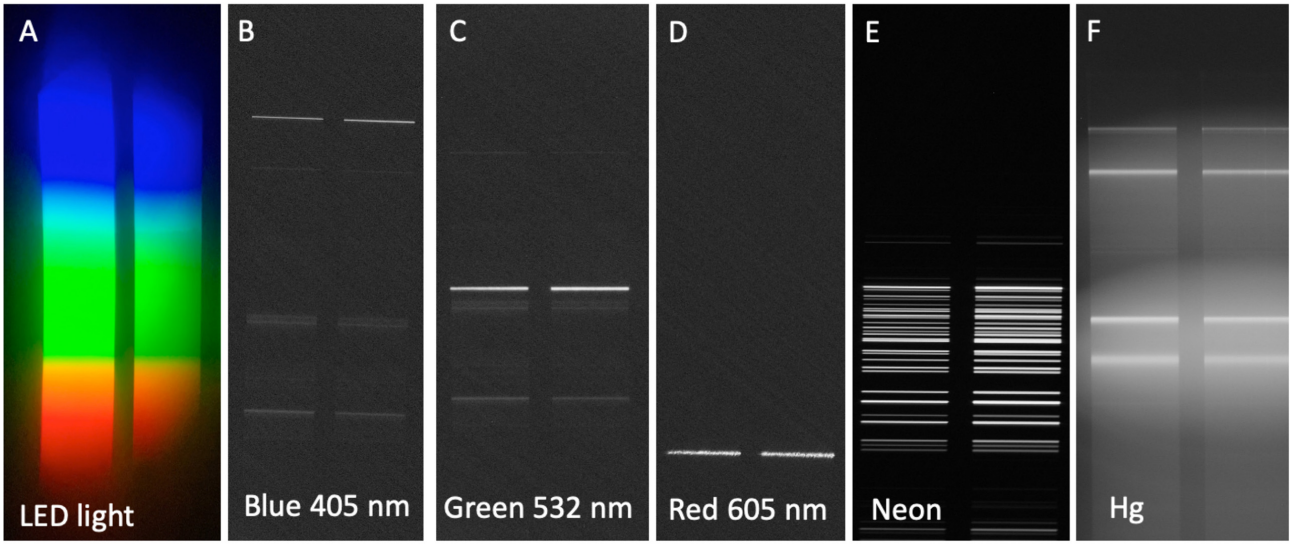

Fig. 2. Light spectra. Light spectra of normal LED light (A), the 3 laser blue (B), green (C), and red (D), and the neon lamp (E) and mercury Hg lamp (F).

2.4 Treatment of Slices and Histological Staining

To induce oxidative stress, slices were incubated for two days with H_2O_2. Briefly, 1 µL of a fresh 30% H_2O_2 solution (Merck, Darmstadt, Germany, K52245709 009) was added to 1 mL medium (9.7 mM final), 1 and 2 days before analysis. To distinguish between morphologically viable or damaged cells at the cellular level, slices were stained with propidium iodide (PI). Briefly, fresh living slices were incubated with PI (2 µg/mL) in medium for 1 h at 37 °C. Following incubation, slices were washed 3 × 3 min with medium, analyzed with the DADOS, and then fixed with paraformaldehyde for 3 h. To show cellular nuclei, post-fixed slices were incubated with the blue fluorescent nuclear dye, DAPI (1:10,000 diluted in T-PBS), for 1 h and then washed 3× in PBS.

For immunostainings, slices were permeabilized (0.1% Triton in PBS 30 min), washed, and blocked (20% horse serum/0.2% bovine serum albumin 30 min), washed, and incubated with primary antibodies against astroglial GFAP (Merck, Darmstadt, Germany, AB5541, 1:2000) or

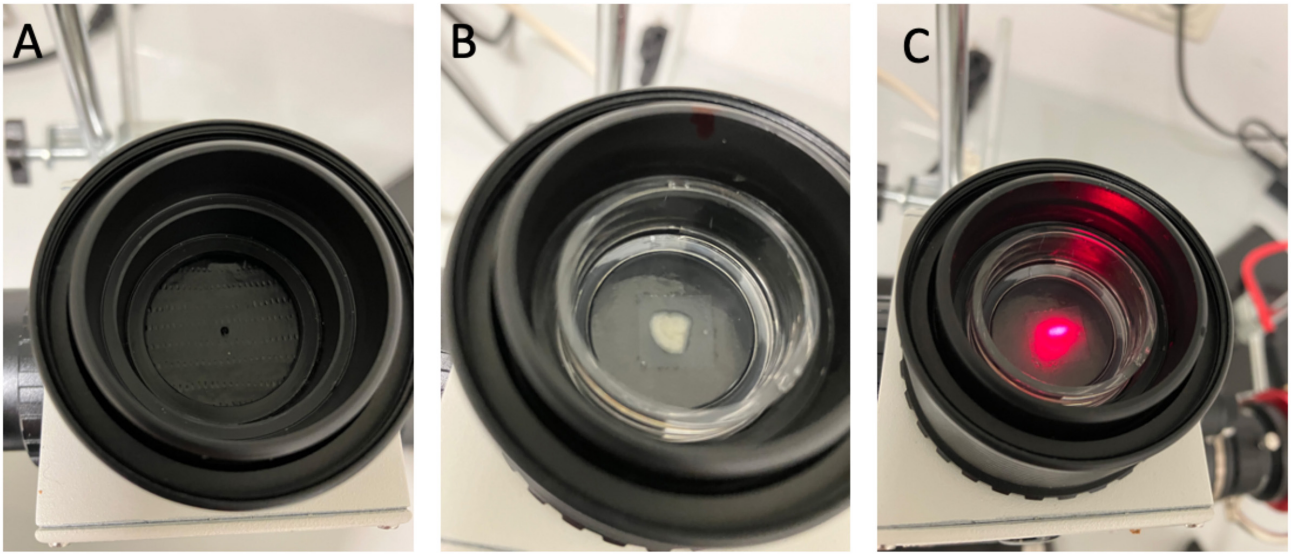

Fig. 3. Measurement of a slice in a special, modified adapter. The adapter has a small 1 mm hole and a height of 2 cm (A). The 300 µm thick quarter slice is positioned over the hole with the hippocampus (B) and stimulated with light, in this example with the red laser (C).

vessel laminin (Sigma L9393; 1:500) for 48 h at 4 °C. After incubation, slices were incubated with anti-chicken (GFAP) or anti-rabbit (laminin) Alexa 488 antibodies, washed, and visualized under the fluorescence microscope (Olympus BX61, Olympus Corporation, Shinjuku City, Tokyo, Japan).

2.5 Analysis Using DADOS and Quantifications With Mygels

To measure the spectrograms, the living brain slice was taken randomly from the incubator in its 6-well in medium and immediately positioned without medium in the special adapter with the hippocampus over the hole. Then the slice was exposed to the red laser, next to the blue laser, then to UV light and then to infrared light with an exposure time of 1 second. Next, the slice was exposed to the green laser for 0.1 seconds and finally to neon for 60 seconds. The entire procedure took no longer than 5 minutes and did not damage the slice. The slice was then put back into the medium and inserted into the incubator and another slice was tested. As soon as all slices were tested, the mercury lamp was started and each slice was again exposed for 10 seconds. The distance of the lasers to the slice was 24 cm, from the mercury lamp 60 cm and from all other light sources 0 cm, plus the 2 cm height of the adapter. The angle of the light source was always 0° directly from above. All exposure times were tested and optimized to give a strong signal. The pictures were saved on the tablet and the JPG files were processed in Affinity Photo 2 (Version 2.6.3., Pantone LLC, Carlstadt, NJ, USA) and inverted from black to white. Next, after corrections to their positions (without changing the contrast or light), the lanes were positioned in a PPT file. For every analysis, a calibration curve was established with the three lasers (Fig. 4A). After saving as a JPG, the spectrogram was analyzed using MyGels (Telethon K.k., telethon.jp) on an iPad generation 9 with OS18.3.

The “MyGels APP” application was opened, and the saved JPG was opened as a new gel, adjusted, and after automatic lane detection, was saved. Next, the 3 lanes with the lasers (405, 532 and 605 nm) were overlaid (Fig. 4A) and then a calibration line was generated (Fig. 4B), giving a spectrogram (Fig. 4C). All spectrograms were analyzed based on this calibration.

Statistical analysis was performed by one-way ANOVA with a subsequent Dunnett post hoc test, compared to the controls. For H_2O_2+PI values were compared against H_2O_2. A p-value < 0.05 was considered significant. In all cases, n $\geq$8 mice were tested. The slices were randomly distributed to the groups. Values are given as mean±SEM. All quantifications were performed in a blinded manner. For the analysis, KaleidaGraph V5 (Synergy Software, 2457 Perkiomen Ave, Reading, PA 19606, USA) was used. Normality was not formally tested. Homogeneity of variance was confirmed by residual plots in previous experiments under the same conditions. If the assumptions had been violated, a Kruskal–Wallis test with Dunn's post hoc test would have been used.

3. Results

3.1 Spectrograms of Brain Slices Stimulated With Seven Light Sources

The 300 µm brain slice was taken freshly from the incubator, immediately measured, and the light exposure was optimized in order to receive a strong spectrogram band. The red and blue lasers gave a single lane at approx. 605 nm

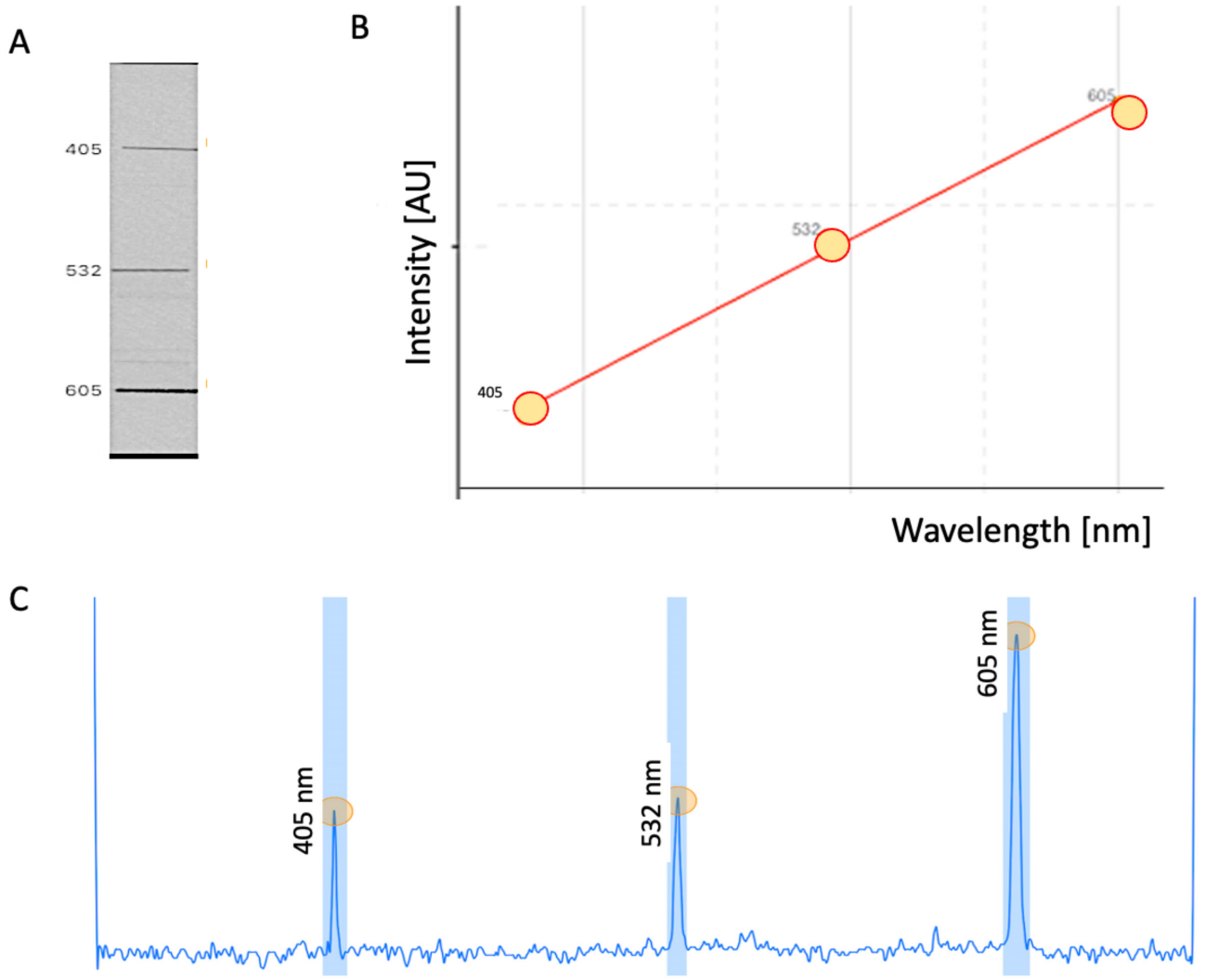

Fig. 4. Analysis of the spectrograms using MyGels. A spectrogram with all 3 lasers was overlaid (A) and measured with MyGels, and a calibration line was processed with these 3 lanes (B). An example of the spectrogram for the 3 lasers is shown in (C).

and 405 nm, respectively, when exposed for 1 second (Fig. 5). With the green laser, the slices were exposed for 0.1 sec and gave a signal at approx. 532 nm (Fig. 5). Slices were exposed to UV and IR light for 1 sec each and gave a single band at approx. 405 nm (UV) or 600 nm (IR) (Fig. 5). Slices were exposed to neon for 60 sec, and several bands >530 nm were seen (Fig. 5). When slices were exposed to mercury (10 sec), four prominent bands became visible at 412, 435, 520, and 547 nm (Fig. 5).

3.2 Microscopic Evaluation of the Slices

To ascertain the morphological quality of the slices, the brain slices were fixed in paraformaldehyde for 3 h after the spectrography. Slices were then evaluated under the light microscope and showed the typical morphology of the hippocampus (Fig. 6A). After staining with blue fluorescent nuclear DAPI, a homogeneous cell layer was visible (Fig. 6B). No signal was seen in the green (Fig. 6C) or red channel (Fig. 6D) of controls. Treatment of living slices with H_2O_2 and labelled with PI for 1 h showed strong red fluorescent nuclear staining of morphologically damaged brain cells (Fig. 6E). Immunostaining for astroglial GFAP showed severely damaged astrocytes after H_2O_2 (Fig. 6F–H), while laminin+ vessels were quite stable (Fig. 6I–K).

3.3 Quantitative Effects

All quantitative data are given in the **Supplementary Tables**. Fig. 7A shows the wavelengths (mean±SEM) of the 7 light sources in control slices ordered by wavelength and color. Using the mercury lamp, 4 prominent lanes were found at 412 nm, 435 nm, 520 nm, and 547 nm. Using the neon lamp, 8 lanes were clearly detectable at the following wavelengths: 554, 579, 603, 612, 629, 654, 665, and 686 nm. For mercury only the 520 nm lane and for neon only the 579 nm lane, are shown in the Figures, as these are very intense, representative of the other lanes and are between the green laser (509 nm) and the red laser (618 nm). Fig. 7B shows that short (1 hr) treatment with PI reduced

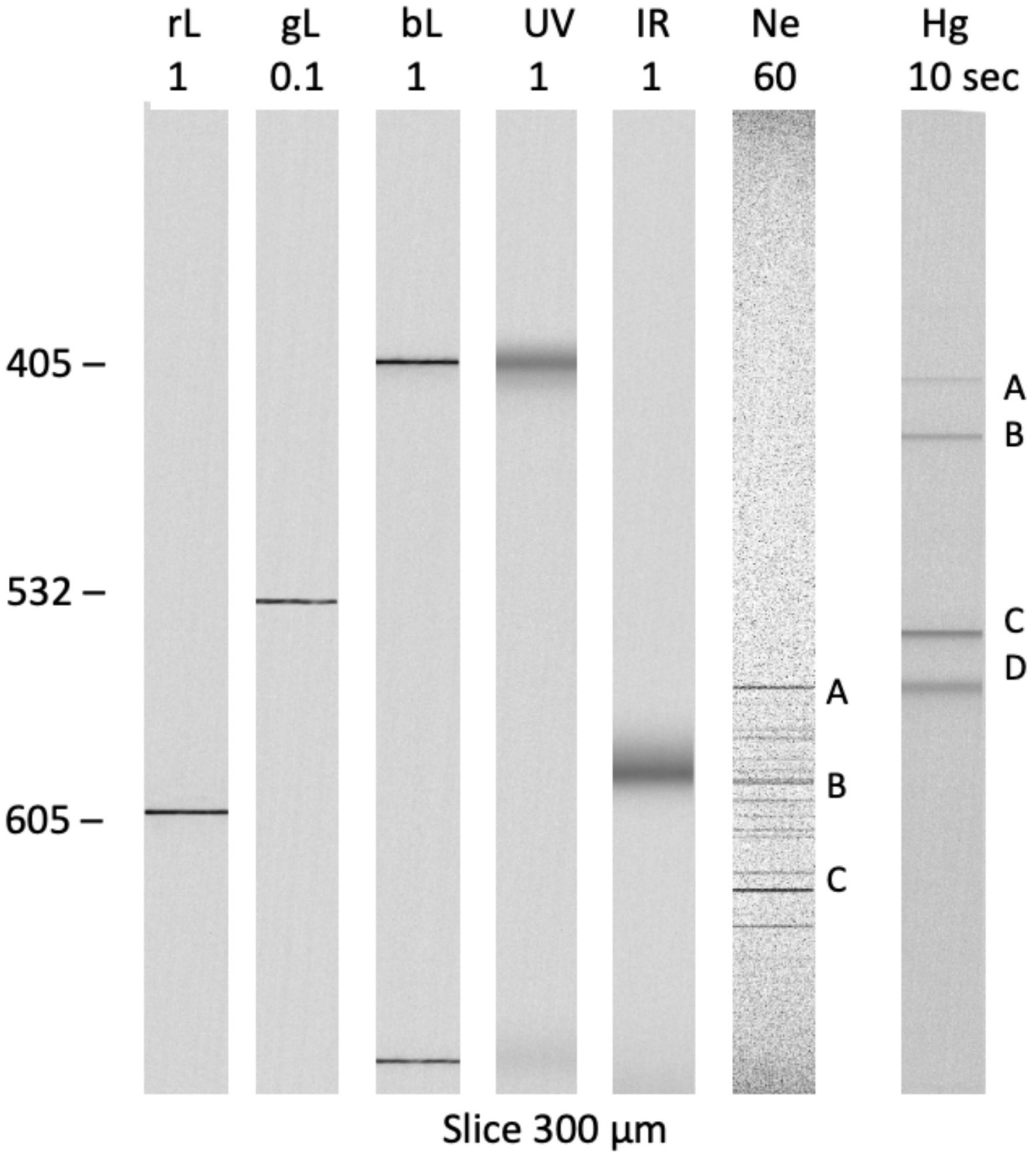

Fig. 5. Slices were measured with seven light sources. The fresh brain slices (300 µm, cultured for 2 weeks) were positioned with the hippocampus in the adapter and exposed to the red laser (rL), green laser (gL), blue laser (bL), ultraviolet (UV), infrared (IR), light or neon (Ne), or mercury (Hg) light. The optimized exposure time is given at the top in seconds. On the left side, the wavelength is given in nanometers. Note that neon is very weak and needs an exposure of 60 seconds.

the wavelengths with mercury exposure (all tested lanes; see **Supplementary Tables**) but enhanced the wavelengths with neon exposure (all tested lanes; see **Supplementary Tables**), but had no effect with the other 5 light sources.

Treatment of slices with hydrogen peroxide significantly reduced the wavelengths when exposed to infrared and red laser (Fig. 8A). Hydrogen peroxide enhanced the peak area (intensity) when slices were exposed to UV, mercury, neon and infrared light (Fig. 8B).

4. Discussion

The present work shows that oxidative stress-mediated processes can be measured with a DADOS spectrograph in living organotypic brain slices. Oxidative stress was induced with H_2O_2, and slices showed a differential spectral pattern when stimulated with seven different light sources.

4.1 DADOS Spectrograph

The DADOS is a slit spectrograph developed at the Max-Planck Institute for Extraterrestrial Physics in Garching [20]. This instrument was initially developed for astronomical visualization, but was adapted in this study to analyze brain slices. A spectrograph analyzes the light spectrum versus intensity, giving a diagram with wavelengths in the visible light, usually between 300–800 nm. In its conventional astronomical use, this provides us with infor-

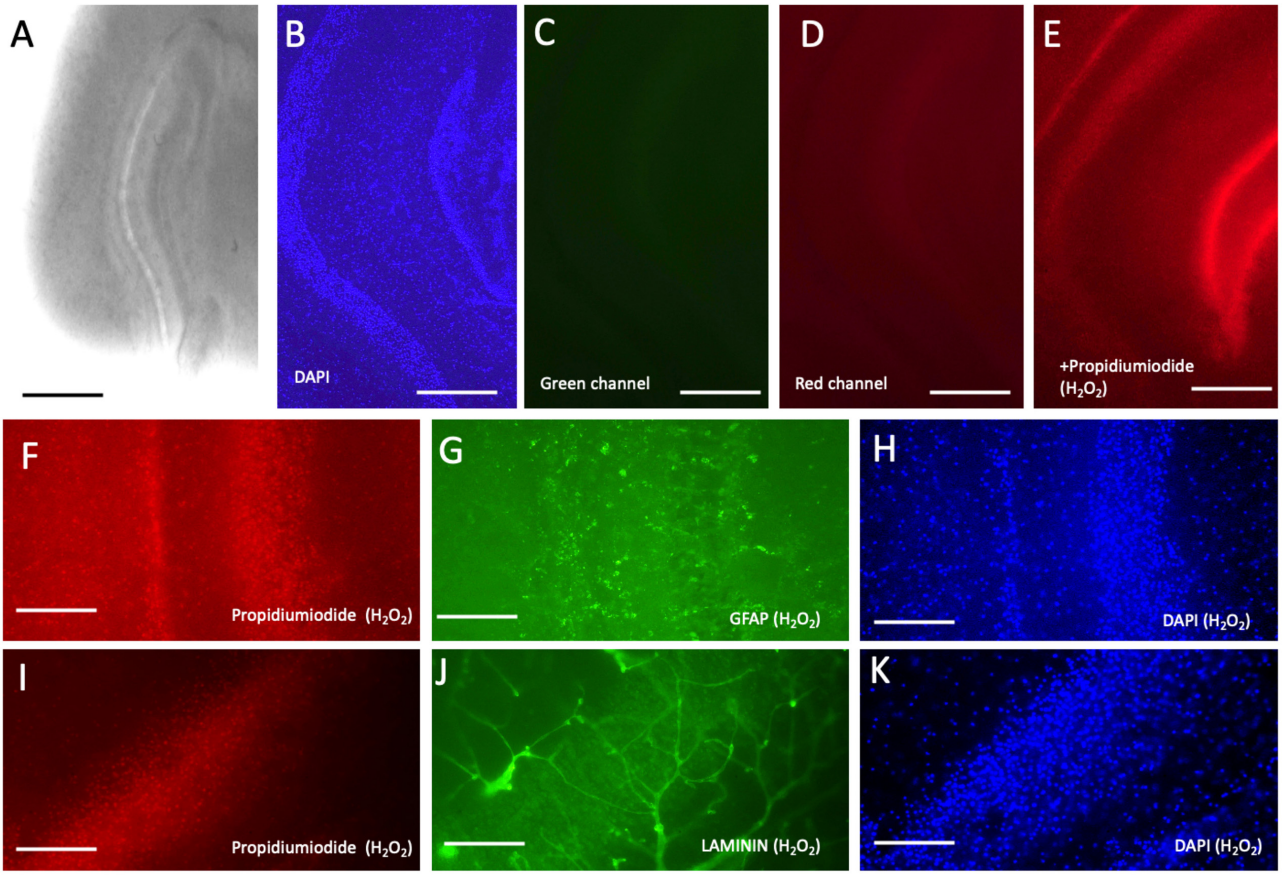

Fig. 6. Morphological analysis of the brain slice under the microscope. The brain slice had a thickness of 300 µm and was cultured for 2 weeks and visualized in a light microscope, showing the typical hippocampal structure (A). After spectrography, slices were fixed in paraformaldehyde and stained with the blue fluorescent nuclear DAPI (B). No background signal was seen in the green (C) and red (D) channels. Staining of slices with propidium iodide shows strong red fluorescent nuclei, pointing to cell death (E). Immunostaining was performed for the astroglial marker glial fibrillary acidic protein (GFAP, F–H) or vessel laminin (I–K). (F,I) show the same respective PI stainings, (G,J) the green fluorescent GFAP or laminin, and (H,K) the same respective DAPI+ area. Scale bar = 650 µm (A–E) and 150 µm (F–K).

mation on the function of a star or a galaxy millions of light-years away. A spectrograph separates light of different wavelengths with a diffractive element into its spectrum, resulting in a spectrogram. Usually, these lines characterize the elements of a star, especially its movement away from the Earth, seen as a shift toward red or blue light, due to the Doppler effect [23]. In the present study, we favored the hypothesis that an organotypic brain slice can modify the spectrum and thus provide information on oxidative processes in the slice. We hypothesize that H_2O_2 causes a shift in wavelength or intensity when exposed to seven different light sources. The DADOS system was purchased from Baader Planetarium, and the costs are relatively low, with approx. 5000 euros for the complete set-up. The brain slice model has been established in our lab for nearly 25 years [1,2,3], and it was only necessary to couple and optimize the system as a pilot project. In fact, we show that the distinct lanes in the brain slice model can be detected with the DADOS.

4.2 The Brain Slice Spectrum

As we adapted the DADOS, three defined lasers were used for calibration: a red (605 nm), green (532 nm), and blue (405 nm) laser. Each laser gave a clear single band at the respective size; only the blue laser showed an additional (artificial) high red band, which was neglected. Based on the three lasers, a calibration curve was generated, which nearly gave a clear regression line. Based on this calibration, the spectrum of the brain slices was calculated and we found that the quantitative analysis was not identical for the respective laser. The blue laser showed a band of 412 nm (should be 405 nm), the green laser showed a band of 509 nm (should be 532 nm), and the red laser showed a band of 618 nm (should be 605 nm) in control slices. While it could be caused by a slightly incorrect calibration, it is possible that the 300 µm thick brain slices themselves cause a slight shift in the wavelength to the red spectrum (+7 nm for blue, +23 nm for green, and +13 nm for red).

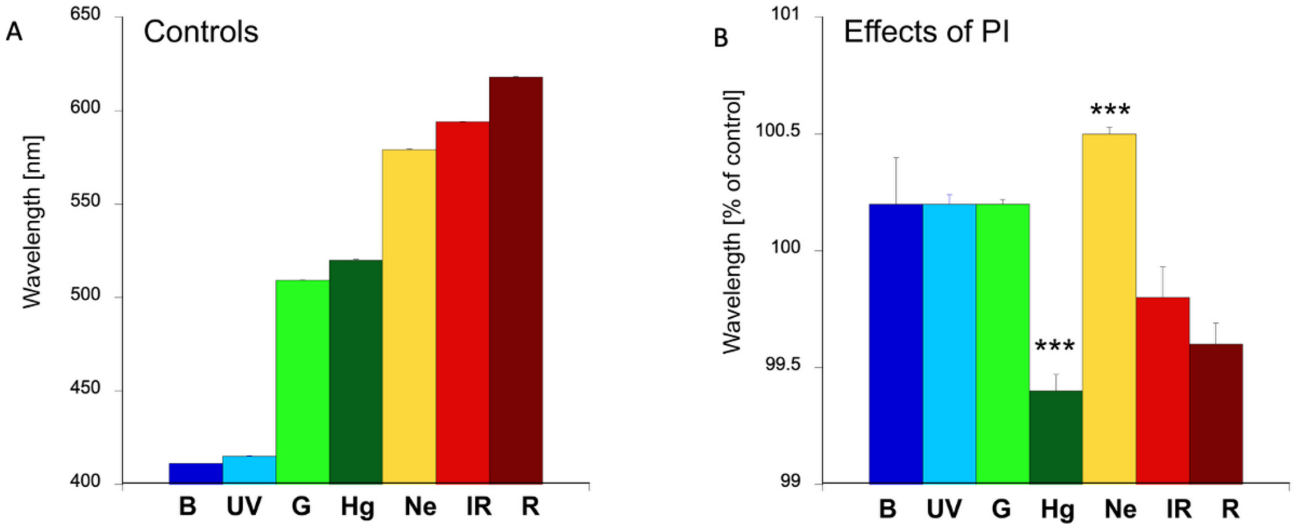

Fig. 7. DADOS spectroscopy of slices in controls and effects of propidium iodide. (A) shows the wavelengths of the 2-week-old control slices tested with all 7 light sources and ordered by wavelength in color. For mercury only the 520 nm lane and for neon only the 579 nm lane are shown. (B) shows the effects when some control slices were exposed to propidium iodide (PI) for 1 hour just before analysis. All values are given as mean ± SEM, and statistical analysis was performed by one-way ANOVA with a subsequent Dunnett post hoc test (*** $p < 0.001$ versus controls). The number of tested mice was n = 12 (controls) and n = 8 (PI). Abbreviations: B, blue laser; UV, ultraviolet; G, green laser; Hg, mercury; Ne, neon; IR, infrared; R, red laser.

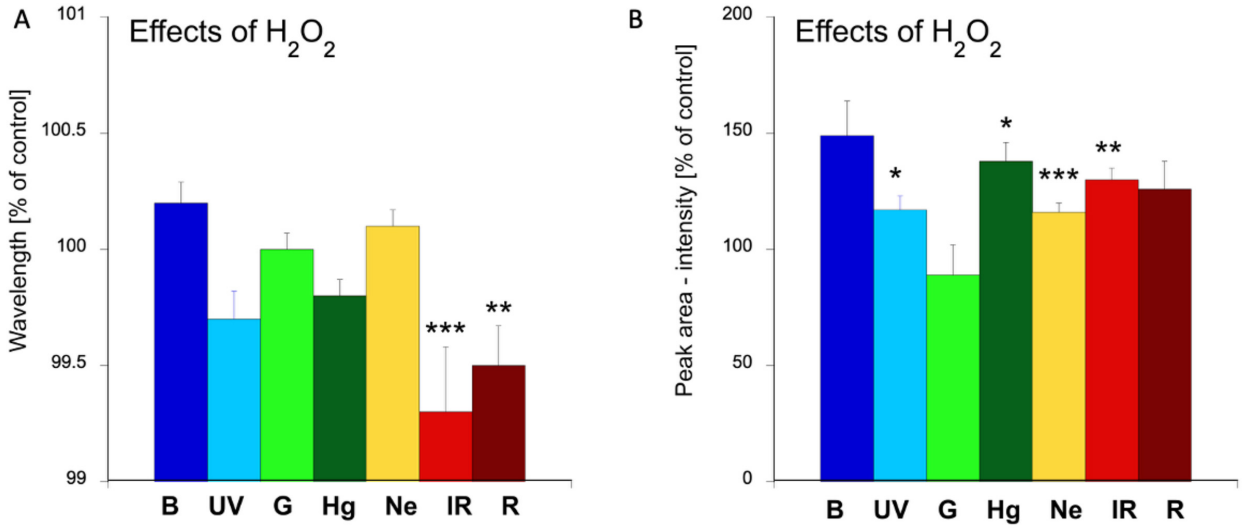

Fig. 8. Effects of hydrogen peroxide (H_2O_2) in living brain slices on wavelengths (A) or peak area = intensity (B). The 7 light sources are ordered by wavelength in color. For mercury only the 520 nm lane and for neon only the 579 nm lane, are shown. All values are given as mean ± SEM, and statistical analysis was performed by one-way ANOVA with a subsequent Dunnett post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The number of tested mice was n = 11–12. Abbreviations: B, blue laser; UV, ultraviolet; G, green laser; Hg, mercury; Ne, neon; IR, infrared; R, red laser.

4.3 Effects of H_2O_2-Induced Oxidative Stress on Wavelength and Area

As aforementioned, we cannot exclude a slight shift in the calibration caused by the brain slice itself, but all changes within the identical model are correct. As all handling was done the same way, including the same calibration, we can clearly show that an H_2O_2-induced metabolism causes a shift in the wavelength. Our data show that H_2O_2 altered the spectral profile when exposed to oxidative stress. It reduced the wavelength with infrared and red laser light, which was reproducible. More importantly, oxidative stress also enhanced the peak area (intensity) when slices were exposed to UV, mercury, neon and infrared light. In order to evaluate any effects on cell morphology, we tested the red fluorescent dye PI. PI is a major optical confounder, as it

is a fluorescent molecule and it can enter damaged cells. We found that PI alone altered the spectral image but only when exposed to mercury or neon, which must be considered in future studies. While PI is a marker for dying cells, we do not call these cells healthy or dead, but rather as morphologically viable. However, the hydrogen peroxide effect was fully reproducible for the wavelength changes in the presence of PI, but only with neon (see **Supplementary Tables**). Regarding changes in the peak area, the effects with PI must be taken with care and should be followed up better in future studies (see **Supplementary Tables**). Anyhow, hydrogen peroxide at a high concentration of 9.7 mM for 2 days definitely causes oxidative stress and possibly also damages cells. The use of 2 immunological markers laminin and GFAP, is not sufficient to determine cell death and more cell death-specific markers (e.g., neurofilament, MAP-2, or neuron-specific markers, or Iba1 for microglia) must be tested. In order to show functional integrity, electrophysiological evidence should also be obtained.

4.4 What Can the Brain Slice Spectrum Tell Us?

The idea of using the DADOS system for spectroscopic analysis of living brain slices arose from the primary cellular biology protocol of fluorescence microscopy. The principle is that light (e.g., from a mercury lamp) at a specific wavelength excites cells labelled with a secondary antibody (e.g., Alexa-488, blue light) and is emitted with a longer wavelength and detected as green light (e.g., at 520 nm) and visualized. Many fluorescent dyes are commercially available, ranging from blue to far-red fluorescent. Such a dye is also the blue fluorescent nuclear DAPI stain (EX 360 nm and EM 462 nm) or the red fluorescent PI (EX 533 nm and EM 620 nm) to detect cellular processes. In the setup, we started with the microscopic mercury lamp with the DADOS, and soon recognized that a simpler optimization with single lasers needed to be done first. So, this setup resulted in the results of testing seven different light sources with defined exposure times.

For the pilot study, the setup was first to test the spectral effects on brain slices alone, second to test H_2O_2 induced oxidative stress, and third to evaluate changes with the red fluorescent dye PI, which penetrates only dying cells. First, we could visualize a spectrum in living control slices with all light sources, which gave a clear reproducible result when using a high number of mice. Second, the H_2O_2-induced oxidative stress caused a shift in wavelength and increased the intensity of the signal. Third, PI treatment altered the spectrum when exposed to mercury or neon and any combined effects must be considered with care. Definitely, DADOS spectroscopy is a good tool to determine oxidative stress processes in living organotypic brain slices. But what does the spectrum tell? Light spectra allow the visualization of the status of tissue and cells, and inform researchers on the chemical composition or functional changes caused by light changes. Three different light changes may affect the tissue: first, light absorption, which means the uptake of light into the cells; second, light emission, which means the uptake and subsequent light output; and third, the light reflection on the cells. In the present study we cannot show which cellular effects occur in the brain slices, but we suggest the following mechanisms: (1) the spectrum may give information on the energetic activity possibly involving mitochondria, (2) on the concentration of molecules, (3) on the chemical composition of molecules, (4) on the oxidative balance of cells, (5) on the lipid metabolism, (6) the content of oxygen and the uptake into cells, (7) on the sensitivity to light for different brain cell types or (8) on different metabolic activities reacting different to wavelengths. Although the shift in wavelength is very, very low, we reproducibly detect changes with at least 8–12 brain slices from different mice.

4.5 Limits and Outlook

The acknowledgement of the many limitations of the study is crucial. (1) As mentioned, the main limit is that we cannot determine the cellular effects seen with wavelength shift and signal enhancement. Much work is necessary following this starting point. (2) As mentioned above, usually slices with a thickness of 150 µm are the best model, as they flatten and become transparent. In the present study, we decided to start with 300 µm thick slices, as we thought that this might provide better results, since we did not have any experience before. These thick slices also did not give optimal results in the immunostaining, as the antibodies do not penetrate well into thick tissues. In the next step, thinner slices should be tested with the DADOS. (3) As for reproducibility, we used a high number of slices (n = 8–12 mice) and are confident that the effects are reliable and significant. The only problem we identified is that the cut-out of the spectra and positioning in the PPT file could cause some shifting of the spectra. As the changes are very low, this could cause some variability. (4) Next, we focused on the hippocampus and positioned the hippocampus directly over the 1 mm hole in the adapter; also, here, some variations in position may cause differences. (5) Technically, the light could slightly differ in angle (standard 0°) to the brain slice; otherwise, this may cause a shift in wavelength. As slices have nearly the same thickness, and the light source is not moved, and slices are always placed the same way, a change in the light angle should be excluded.

As an outlook for the future, we reiterate that this pilot study potentially unlocks a pioneering field in visualization of biological samples. To the scientific community, we suggest the following: (1) We will perform a dose-response curve for hydrogen peroxide to better explore the mechanisms in the wavelength changes, block the effects with antioxidants, and use more specific drugs to induce oxidative cell death. (2) We will prove cell death and induce cell death also with inflammation, trauma or ischemia and test other drugs or toxins. (3) We will test various other com-

mercially available fluorescent markers at varying wavelengths, as our preliminary results point to luminol as a promising dye. (4) We aim to study disease-related models, especially Alzheimer's disease, studying the beta-amyloid and tau depositions in transgenic mice. (5) The functional effects of the wavelength shift must be evaluated. So far, we do not know what the wavelength decreasing or increasing means, whether this is a change in the elements, the membranes, or the cell structure, or perhaps a molecular effect. (6) We also do not know why some lights enhance the intensity (as measured by peak area). Are there some structures that specifically react to light linked to oxidative stress? (7) Do different brain cells react differently, e.g., neurons or glia, or vessels? (8) Can we measure specific changes in energy activity or lipid metabolism, or other relevant molecular processes?

5. Conclusion

Taken together, our data show that oxidative stress-mediated processes can be measured with a DADOS spectrograph in living organotypic brain slices. The slices showed a differential spectral pattern when stimulated with seven different light sources. This study pioneers a novel tool of cheap and easy spectroscopy for living brain slices.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Writing, figures, experiments, conceptualization, and final approval: CH. CH has participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments (taking an organ) were approved by the Austrian Bundesministerium Bildung, Wissenschaft und Forschung, under the approval number 2021-0.150.227 (approved on August 26, 2021). All animal experiments were carried out in compliance with the Austrian Animal Experiments Act (Tierversuchsgesetz 2012), which transposes EU Directive 2010/63/EU into national law. The study was performed in accordance with the ARRIVE guidelines and the 3Rs principles (Replacement, Reduction, Refinement).

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Conflicts of Interest

The author declares no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL54348.

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